PICOSECOND KINETICS OF THE 1250 nm BAND OF THE *RPS. SPHAEROIDES* REACTION CENTER: THE NATURE OF THE PRIMARY PHOTOCHEMICAL INTERMEDIARY STATE

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1. Introduction

The reaction center protein of *Rps. sphaeroides* includes four bacteriochlorophylls (BChl) and two bacteriopheophytins (BPh) (see [1–3]). When the reaction center is in its oxidized state, EPR [5] and ENDOR [6] studies show that the cationic free radical may be delocalized over two of the BChls. EPR studies on the light excited reaction center triplet or 'biradical' state are consistent with this state also being contained within a dimer structure [7,8]. The final products of the light reaction within the reaction center can be represented as [BChl $\overset{+\cdot}{-}$ BChl] X^-, comprising the oxidized BChl dimer (half reduction potential (E_m) ≈ + 450 mV; pH 7.0) and a reduced primary acceptor (X) which is very probably a ubiquinone–iron compound (the E_m of X/X^- in the isolated reaction center protein approximately –45 mV; pH 7.0). (See reviews [1–4] for further details and references).

Picosecond spectroscopy in the 500–700 nm region suggests that an intermediary state in the BChl/BPh complement is formed in ⩽ 10 ps [9,10]. The properties of this rapidly formed state are not consistent with those of a fluorescent or pure singlet excited state (see [9–11]), but there is insufficient information to make any firm alternative assignments. The decay half-time of the state when normal photochemistry can proceed is 100–150 ps [9,10] and this has been interpreted as being the rate of electron transfer from the intermediary state to X since the spectrum remaining after this time resembles that of the oxidized reaction center. If X is chemically reduced before activation so as to block the final electron transfer reaction to X the intermediary state designated P^F [12] lasts for approx. 10 ns [12].

The suggestion [5,13] that there might be an intermediary-electron transfer state within the BChl/BPh complement (see also [14]) before the electron transfer to X, is compatible with the spectrophotometric properties of the intermediary state, P^F, [9,10,12] and the reaction center triplet or 'biradical' EPR signal [7,8] may be important to this consideration.

In the hope of gaining unequivocal information on the chemical nature of the intermediary state we have examined the 1000–1350 nm spectroscopic range in the picosecond time domain. The oxidized reaction center exhibits an absorption maximum at 1250 nm (extinction coefficient 14000 M^{-1} cm^{-1}) (see [1]), an absorbance (see fig.1) which is not apparent with the monomeric BChl in organic solution [15,16]. Consequently the 1250 nm absorbance is unique to the oxidized reaction center BChl dimer and this could tell us at which point the oxidized dimer is formed. If the oxidised dimer is part of the intermediary state the 1250 nm absorbance will appear in ⩽ 10 ps; if not the 1250 nm rise kinetics should be 100–150 ps as X becomes reduced and the dimer becomes oxidised. This paper examines these possibilities.

2. Materials and methods

The experimental system utilized to measure the 1250 nm band behaviour in the picosecond time

domain is similar to that described in detail previously [17,18] except that a germanium diode replaced the vidicon. 530 nm excitation was generated by a frequency doubled Nd/glass laser (1060 nm). The changes in optical absorbance in the 1000–1350 nm region were observed by a picosecond duration continuum generated from the 1060 nm Nd/glass laser via carbontetrachloride in a 20 cm optical path cell with appropriate filters to remove the excitation beam and 1060 nm light.

Reaction centers were isolated by the lauryldimethylamide-*N*-oxide (LDAO) method described (see [1]) except that purification was done with a DEAE column chromatography during which LDAO was replaced by Triton X-100 (0.1%). Redox potentiometry was performed as previously described [10,19].

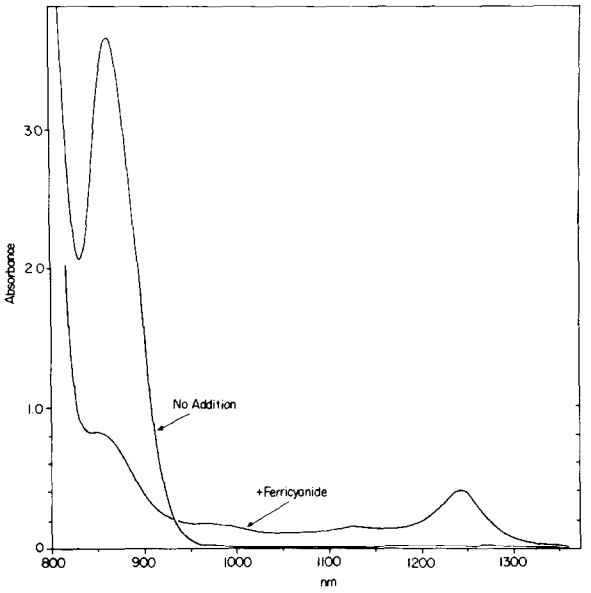

Fig.1. Absorption spectra of reaction centers from *Rps. sphaeroides* in the neutral form and oxidized from in the near-infared. Reaction center concentration was 140 μM in 50 mM Tris–HCl buffer pH 8.0 containing 0.1% Triton X-100. The spectrum reflects the absorbance of the material in a cuvette of 2 mM light path, as used for the picosecond kinetic analysis. For technical reasons the spectrum was taken in a 1 mM light path and multiplied by two. For the oxidized form the redox potential (E_h) was $\sim$ +520 mV established with 1 mM potassium ferri/ferrocyanide.

3. Results and discussion

Fig.1 shows the spectra of the reaction centers in the near infrared in the neutral state [BChl——BChl] X and in the chemically oxidized state [BChl$^{+\cdot}$—BChl] X. Fig.2 shows kinetic features of the flash induced absorbance changes seen at wavelengths near 1250 nm in reaction centers under different conditions. With the reaction centers in the neutral [BChl ——BChl] X state before activation an absorbance increase maximal at 1250 nm is formed following the flash (open circles). The measured absorbance changes fit well on the spectrum characteristic of the chemically oxidized reaction center from 1000–1370 nm (dashed line). However, as shown on the right of fig.2, the band is formed within the $\sim$ 10 ps instrumental resolution time and thus does not rise with the half-time of 100–150 ps which would be encountered if the 1250 nm band was formed as the reaction center became oxidized simultaneously with X reduction. We may conclude that the 1250 nm band, and therefore, the oxidized dimer is also part of the reaction center intermediary state which is the precursor to the final [BChl$^{+\cdot}$—BChl] X$^-$state. Appropriate to this conclusion chemical reduction of the primary acceptor to form [BChl——BChl] X$^-$before activation does not prevent the flash induced formation of the 1250 nm band. (Fig.2: solid squares, measured 20 ps and 50 ps after the flash; see Figure legend for details of the chemical reduction). The points fit well on the dashed line representing the chemically oxidized state.

Prior chemical oxidation of the reaction center with ferricyanide to form [BChl$^{+\cdot}$—BChl] X before activation (i.e. the 1250 nm band is already formed as shown in fig.1) yields no significant flash induced absorbance changes at 1250 nm. (fig.2; open triangles, measured after 50 ps and 1 ns). This is in keeping with the requirement for the BChls to be in the neutral state for normal reactions to proceed and provides further identification of the 1250 nm band as originating in the activation of this neutral state.

The data are consistent with steps 1–3 in the working scheme shown in fig.3. The observation of the 1250 nm band within 10 ps after activation and independent of the prior state of reduction of X is compelling evidence that an electron has left the dimer during the formation of the intermediary state. The

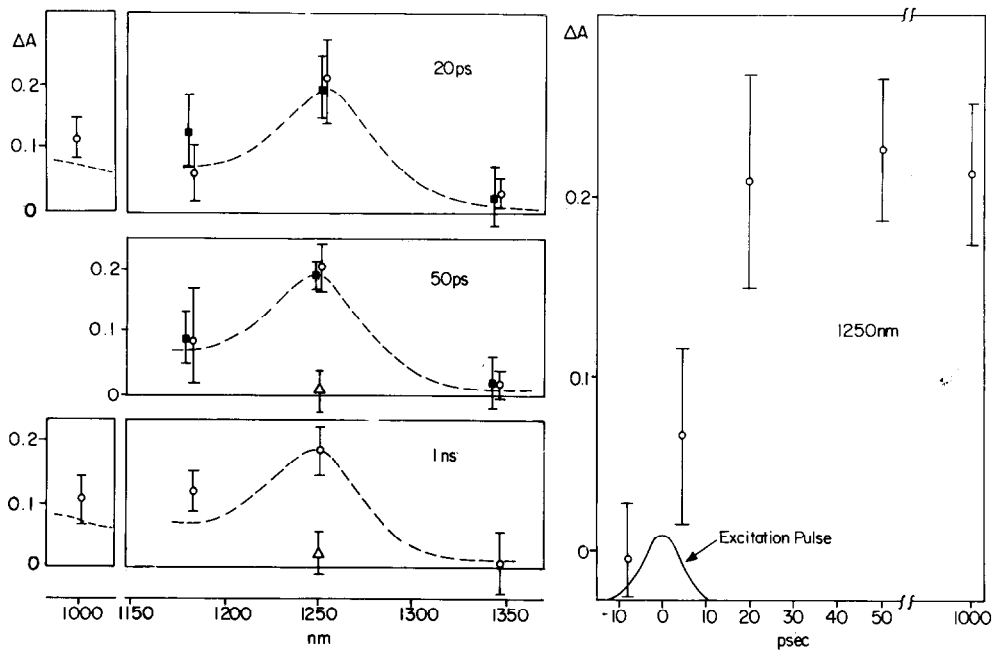

Fig.2. Picosecond spectrophotometric changes in the near infrared region of *Rps. sphaeroides* reaction centers. The reaction centers were used and suspended as described in fig.1 and examined in a 2 mM anaerobic redox cuvette. The reaction centers were poised at different redox states as follows: In the neutral form, BChl —— BChl X (○); in the ferricyanide oxidized form, BChl $\overset{+\cdot}{——}$ BChl X, (△); and with the primary electron acceptor reduced, BChl —— BChl X^-, (■). In the letter proof that the primary acceptor was reduced was obtained by taking a sample of reaction centers anaerobically. For EPR analysis at liquid helium temperatures. This showed (a) the fully reduced X^- at g = 1.82 in the dark and (b) no light induced g = 2 signal, but instead the reaction center triplet/biradical signal was apparent (see [19]). The right of the figure shows points taken at various times before and after the flash at 1250 nm. On the left are shown points taken at various wavelengths and times after the flash. The points are the average of at least four determinations. The error bars represent the mean standard deviation. The laser light used for activation was not saturating.

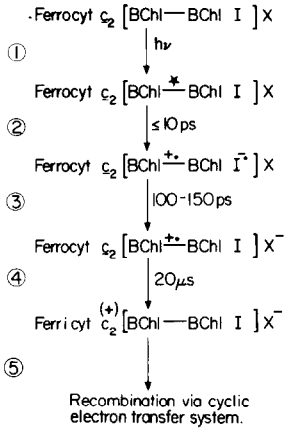

Fig.3. Simple working scheme for the early forward steps of photosynthesis.

intermediary state (P^F) could then be a composite of the oxidized BChl dimer and reduced component symbolized by 'I' for intermediate.* This raises the question as to the nature of I, and clearly the other BChls or BPhs of the reaction center are prime candidates. Of these two possibilities we have tested spectrophotometrically for location of the electron on a BChl (i.e. $BChl^{-\cdot}$). Monomeric $BChl^{-\cdot}$ in organic solvents such as dimethylformamide has an absorption at 1000 nm [20]; the extinction coefficient is $\sim$ 16000 M^{-1} cm^{-1}. Examination of the reaction centers at 1000 nm (fig.2 left) reveals no absorbance increase at this wavelength which is not already ascribable to that of the oxidized cation

* I is defined as a chemical species which may take part in the initial photochemical process and act as an electron transfer intermediary between the BChl dimer and X.

radical of the BChl dimer (dashed line); the changes observed at 20 ps where the anion radical would be fully formed, and at 1 ns where it would have disappeared, are essentially the same. This is evidence against the existence of $BChl^{-\cdot}$ as a clearly identifiable part of the electron transfer state, assuming that the in vitro spectrum [20] is relevent to the reaction center protein. An alternative location for the electron is on BPh, since in this case the anion radical of BPh ($BPh^{-\cdot}$) does not absorb beyond 950 nm [16]. In this respect Fajer et al. [16] have produced information regarding the possibility that location of the electron may be on BPh. In combining the expected optical changes which would occur on chemical oxidation of the reaction center [BChl——BChl] → [BChl$^{+\cdot}$—BChl]) with those occurring on reduction of isolated monomeric BPh in dichloromethane (BPh → $BPh^{-\cdot}$), they have revealed a spectrum which resembles that reported for the P^F spectrum from 400–1400 nm (derived from [9,10] and this work).

In preliminary experiments we have employed redox potentiometric techniques to reduce the postulated intermediary state component chemically to form $I^{-\cdot}$ and thus attempt to prevent the photochemistry from proceeding beyond the excited singlet state (fig.3, step 1). Use of methyl and benzyl viologens to establish redox potentials of −430 mV at pH 10 did not diminish the formation of the 1250 nm band following a flash. The measured E_m values of BPh/$BPh^{-\cdot}$ and BChl/$BChl^{-\cdot}$ in organic solvents are estimated (with respect to the standard hydrogen electrode) to be ∼ −550 mV [16] and −860 mV [15] respectively although how close these E_m values are to those encountered in the reaction center is of course open to conjecture. However, although not negative enough to test the above candidates, (we also do not know the reactivity of the dyes with I), we may tentatively expect the E_m of $I/I^{-\cdot}$ to be significantly more negative than −430 mV, consistent with the considerations dealt with more fully by Fajer et al. [16]. As such the incident light energy (1.4 eV at 870 nm) will be converted in the proposed [BChl-$^{+\cdot}$—BChl $I^{-\cdot}$] intermediary state into possibly over a volt of redox potential energy (see also fig.1).

3.1. *Role of I in the light driven forward reaction*

If the above considerations are correct, the half-reduction potential difference (ΔE_m) between $I/I^{-\cdot}$ and $X/X^{-\cdot}$ is large and would strongly favor the complete reduction of X. Even in the natural membrane where the E_m of the $X/X^{-\cdot}$ couple is −180 mV [24] (in contrast with the apparent −50 mV value in the isolated reaction center [19], the ΔE_m could be > 250 mV. On a kinetic basis, the finding that the [BChl$^{+\cdot}$—BChl $I^{-\cdot}$] state (P^F) has a half lifetime about 10 ns [12,21] compared with that of 100–150 ps required for the $I^{-\cdot}$ to X reaction is adequate to secure the virtually complete reduction of X following light excitation of the reaction center. Once formed, the back reaction of [BChl$^{+\cdot}$—BChl I] X^- is relatively slow, half time ∼ 20 ms [22,23]. These parameters thus provide a basis for the observed 102 ± 4% for the quantum efficiency of BChl oxidation in reaction centers [25].

3.2. *The role of I in dark reactions*

The possible existence of I permits speculation that the 20 ms barrier to the reversed reaction (cf. [21]) could involve the electronegative intermediate I as an obligatory redox agent for the back reaction for electron transfer between X^- and [BChl $^{+\cdot}$— BChl]. A simple view of the back reaction sequence would be $X \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} I \xrightarrow{k_{-2}}$ BChl —— BChl, in which k_1 is about 0.5×10^{10} s^{-1} (halftime 140 ps) and the E_m value for X/X^- is −180 mV. If the E_m for $I/I^{-\cdot}$ is approx. −540 mV (for instance as in BPh/$BPh^{-\cdot}$; see [16] the k_{-1} rate could be as fast as 0.5×10^{-4} s^{-1} (halftime ∼ 140 μs). An even greater redox potential gap between I and X, say $\Delta E_m = -500$ mV, would yield a rate for k_{-1} which would still not exceed the observed 20 ms half-time for the back reaction. With a k_{-2} rate of about 10^8 s^{-1} for the decay of the [BChl$^{+\cdot}$—BChl] $I^{-\cdot}$ state (P^F) the overall $X^- \rightarrow$ [BChl $^{+\cdot}$—BChl] back reaction would be strongly governed by the $X^- \rightarrow$ I step which is consistent with the directly measured X^- oxidation [23] and [BChl$^{+\cdot}$—BChl] reduction halftimes being the same [22,23]. A direct involvement of I in the $X^- \rightarrow$ [BChl$^{+\cdot}$—BChl] reaction, as in the case of the dielectric relaxation model [14], requires that the activation enthalpy of the reaction be zero or positive in accord with the observed very low temperature dependence of the reaction.

The actual decay pattern reported for P^F (experiments done when X reduction is blocked) includes

decay by fluorescence [11] (see also [1–3]) and another spectroscopically identified species designated P^R [12,21]. At room temperature P^R contributes to P^F decay in only a minor way; at 77°K or below however the quantum efficiency of P^R formation approaches 100% [12]. At the low temperatures and under the same conditions of pre-reduced X, illumination also rapidly generates and EPR signal with a quantum efficiency approaching 100% [26]. The signal represents a strongly spin polarized triplet or 'biradical' state for which zero field splitting parameters indicate its location on a dimer structure [7,8]; again the most likely candidate would be the BChl dimer and it is not unreasonable to identify it tentatively with P^R [4,12,21]. The dimer as a triplet or biradical state (i.e. $[BChl^{+\cdot} \text{——} BChl^{-\cdot}]$) fits as a possible stage in the decay or back reaction of the $[BChl \overset{+\cdot}{\text{——}} BChl\ I^{-\cdot}]$ state (P^F) and could be formed by recombination of an electron from I with the unpaired electron on oxidized BChl dimer. Recombination in this way provides an attractive way to explain the strong degree of spin polarization of the triplet by a mechanism analogous with chemically induced dynamic electron spin polarization (CIDEP; see [27]).

Fig.4 summarizes the kinetics and proposed reaction center states involved in the back reactions for the situation at low temperature. The fragment of the reactions determined when X is pre-reduced (B) is fitted into the situation when X is not reduced before activation (A). The dotted line (1) shows the familiar direct recombination process for the 20 ms halftime. The dotted line (2) represents the above discussed departure from this established thinking in which the 20 ms rate-limiting step takes the back reaction only as far as the $[BChl \overset{+\cdot}{\text{——}} BChl\ I^{-\cdot}]\ X$ stage. Both (1) and (2) are consistent with the requirement for X to be reduced to detect the light-generated proposed $[BChl^{+\cdot} \text{——} BChl^{-\cdot}]$ state. In contrast to the separate course for the back reaction of (1), the alternative, (2), utilizes the reverse of the final forward step and the established back reactions shown in fig.4B.

Fig.3 summarizes the forward progress under physiological conditions into the ATP-yielding cyclic electron transfer system (see [4]).

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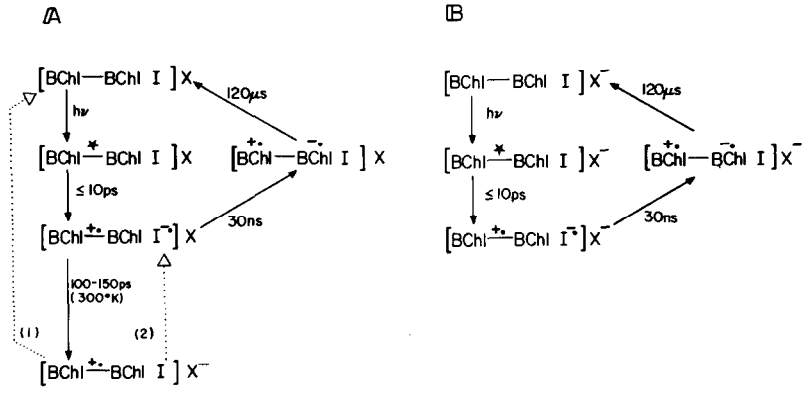

Fig.4. Working scheme for the role of I in back reactions of the primary reactions in photosynthesis at cryogenic temperatures. At low temperatures electron transfer does not proceed beyond step 3 of fig.3, and P^R or the EPR triplet state $[BChl^{+\cdot} \text{——} BChl^{-\cdot}]$ is in the principle route of the back reactions. Part A shows the reactions when X is oxidized and Part B shows reaction when X is prereduced before illumination. The 30 ns and 120 μs steps are the 77°K P^F decay/P^R formation and P^R decay respectively [12,21].

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